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13. ABSTRACT ***(Maximum 200 Words)***

The neuropeptide galanin is involved in the control of a number of physiological functions including neuronal development and survival, neuroendocrine responses and mitogenesis. We report that null mutation of the galanin gene produced mice with reduced mammary ductal side branching during puberty and reduced lobuloalveolar development with lactation failure following pregnancy. Galanin and galanin receptor 2 (GALR2) are expressed in the mammary gland and are differentially regulated during mammopoiesis. Mammary transplantation experiments demonstrated galanin does not act via autocrine or paracrine mechanisms in the mammary gland. Galanin knockout mice have decreased levels of secreted prolactin and interestingly were also found to have an increased ratio of phosphorylated to unmodified prolactin. Unmodified prolactin rescued the lactation failure and treatment of wildtype mice with a molecular mimic of phosphorylated prolactin inhibited lactation and alveolar differentiation. These studies provide evidence for an unexpected role of galanin in regulating prolactin phosphorylation and identify a novel regulatory mechanism where the modification state of prolactin induces different physiological effects in the mammary gland. In addition treatment of whole mammary gland explants with galanin resulted in a 3.8 fold increase in the number of lobuloalveoli demonstrating that galanin also has a direct endocrine action on the mammary gland. These data identify several novel functions of galanin during mammary gland development.

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Galanin, a neuropeptide previously thought to be restricted to the central and peripheral nervous system is implicated in the growth control of a number of cell types. Recently we showed that galanin is expressed by human breast cancer cell lines under the control of estrogen and progesterone. In cell lines derived from small cell lung cancer, a tumour which relapses rapidly with an aggressive phenotype, galanin caused rapid mobilisation of calcium, accumulation of inositol phosphate and activation of the MAP kinase isoform p42 via a protein kinase C dependent mechanism, producing increased clonal cell growth in soft agar. By analogy galanin may also be a growth factor for both the normal breast and breast cancer. This proposal has the objective of testing this hypothesis. The specific aims are to examine the role of galanin in normal mammary gland development, human breast cancer and experimental carcinogenesis.

BODY

The research proposal submitted contains the following objectives (in bold print). Our progress to date with each of these objectives is indicated in plain type.

Specific Aim A: Examination of the role of galanin in normal mammary gland development.

1. **The normal expression patterns of galanin and the galanin receptor subtypes will be examined using *in situ* hybridisation of normal mouse mammary glands at the major developmental stages of puberty, pregnancy, lactation and involution.**

   A mouse mammary gland tissue array is currently being assembled by this project and will be ready for use later this year. This will complement our findings by PCR to date. Please see the attached manuscript for results to date.

2. **The expression pattern of galanin and its receptors by the normal human breast will be examined by *in situ* hybridisation of specimens selected from a panel of 103 breast biopsies obtained at reduction mammaplasty (60 biopsies) or from non involved breast obtained at radical mastectomy (43 biopsies).**

The production of breast cancer tissue arrays was not undertaken by the Translational Research Group due to the last minute withdrawal of the pathologist recruited to mark the tissue blocks for core sampling. This aspect of the project has been stalled as a result. Within the last month however a commercial supplier of human breast cancer tissue arrays has been found (Monarch Medical) and a series of arrays has been ordered, which will allow this important aspect of the project to proceed. The translational research group intends to proceed with their tissue array project once a pathologist can be recruited to the project.

3. **Galanin knockout mice, obtained by collaboration with David Wynick and now at the Garvan Institute, will be used to examine mammary development at puberty, pregnancy, lactation and involution, by whole mount and histological techniques, to determine if galanin is involved in mammary development.**

These experiments have been completed. Please see the attached manuscript for results to date.

4. **To determine if the effects defined in A3 are due to the loss of galanin in the mammary gland, or to indirect effects due to the loss of galanin from other endocrine organs, transplant of mammary glands from wild type and knockout animals will be made to endocrinologically normal hosts and examined by whole mount and histochemistry at puberty, pregnancy and involution.**

These experiments have been completed. Please see the attached manuscript for results to date. These experiments showed that galanin plays no autocrine role in mammary development- the cause of the defect in galanin kos must lie elsewhere.

Comparison of the recombination and cleared fat pad experiments showed remarkable differences in the level of ductal side branching achieved in virgin animals. The stroma used in these experiments was from 129 animals in the

different levels of side branching we hypothesised that it was the mammary stromal compartment that underlay this effect, and subsequent experimentation confirmed this. These experiments were submitted for publication, and following the suggestions of our reviewers (and failure to have the work accepted by a highly regarded journal), we undertook a number of further experiments and we will resubmit the manuscript shortly.

The failure to transplant the galanin ko phenotype left two possibilities, galanin plays a direct endocrine role, or galanin plays an indirect role. Experiments examining the direct role are detailed below in 5A. The similarity between the galanin ko and the prolactin receptor ko suggest prolactin as a possible indirect mediator of galanin action in the mammary gland. To test this hypothesis we formed a collaboration with Dr. Ameae Walker, UC Riverside, who has supplied us with prolactin and a phosphoprolactin mimic to administer via osmotic minipump. Our results showed that prolactin administration can rescue the first lactation failure in galanin kos, that the phosphoprolactin mimic can abrogate lactation in wild type animals, and that galanin knockout mice show altered ratios of phosphorylated to non phosphorylated prolactin. Thus two mechanisms of galanin action on the mammary gland appears to reside in the pituitary. First, galanin controls the overall level of prolactin secretion and second galanin controls the phospoprolactin:prolactin ratio, which determines the overall ability of prolactin to modulate mammary development. Please see the manuscript attached.

5. **The possibility of mammary galanin acting as an endocrine factor to influence mammary development will be examined using conditional galanin knockout animals obtained by collaboration with David Wynick. These animals will have only the mammary gland galanin gene ablated. Hormonal profiles will be measured. A single normal mammary gland will be transplanted to these animals and hormonal profiles again measured. The development of the transplants will be assessed at puberty, pregnancy and involution and compared to a normal mammary glands transplanted to normal hosts.**

The conditional galanin ko animals are not yet available from Dr Wynick. As an alternative we formed a collaboration with Dr. Barbarra Vonderhaar, NCI Bethesda to examine the effects of galanin administration in vitro mammary gland development, using an organ culture technique which has been developed in Dr Vonderhaar's laboratory. These experiments showed that galanin exerts a direct effect on mammary gland development. Details can be found in the manuscript. Dr Vonderhaar has supplied us with frozen galanin treated tissue for RNA extraction, and we intend, as a variation in the approved statement of work, to transcript profile these glands using the Affymetrix U74A2/U74B2 and U74C2 chips, giving us access to almost all of the known mouse transcripts. This will identify the galanin induced pattern of gene expression. We have developed considerable expertise in this area, having previously successfully profiled glands from other experimental models.

Specific Aim B: Examination of the role of galanin in human breast cancer.

1. **The panel of breast cancer cell lines used to examine galanin gene expression will be screened for galanin receptor subtype expression by PCR. Cell lines selected on the basis of receptor expression will be treated with galanin and cell cycle phase distribution, cell growth and colony formation will be measured.**

   Examination of galanin and galanin receptor gene expression in the panel has been completed. Please consult the attached manuscript for details. Galanin treatment of cell lines has been abandoned due to technical failures. The use of the mammary tissue supplied by Dr Vonderhaar provides a very suitable substitute for this aspect of the proposal.

2. **Breast tumours selected on the basis of phenotype from a large panel of specimens will be used to determine which cell types express galanin and its receptors by in situ hybridisation. Results will be related to the normal expression patterns defined in A1 and A2, and to tumour phenotype, steroid hormone receptor expression, markers of poor prognosis, proliferative and apoptotic markers.**

This aspect will be pursued using the human tissue arrays supplied by Monarch Medical. We hope to recruit a pathologist in 2002 to allow construction of our own arrays.

3. **The expression of galanin and its receptors by breast cancers will be measured by RT-PCR and correlated with disease outcome in a large panel of breast cancers for which RNA and 74 month average post diagnosis clinical follow-up are available.**

   This will be pursued using the human tissue arrays supplied by Monarch Medical.

Specific Aim C: Examination of the role of galanin in experimental carcinogenesis.

1. **Conditional galanin knockout mice, lacking galanin expression in the mammary glands, will be treated with DMBA, a chemical carcinogen requiring additional hormonal stimulus for full activity. Tumour latency, frequency, histological grade and metastasis will be compared between genotypes.**

   No progress to date. the conditional knockout animals are not yet available

2. **A transgenic mouse expressing galanin under the control of the mouse mammary tumor virus promoter will be constructed and examined for altered rates of tumorigenesis, either spontaneously in virgin and multiparous animals, or in conjunction with DMBA treatment. Tumour latency, frequency, histological grade and metastasis will be compared between genotypes.**

   The construction of a galanin transgenic mouse was delayed until the mechanism of galanin action was better understood. We have now identified a direct action of galanin on the mammary gland, and believe that the best way to proceed toward identifying the mechanism of galanin action is to transcript profile the galanin

of a galanin transgenic mouse, and week seek permission to make this variation to the statement of work.

Discussion

The results to date show that galanin plays indirect and direct roles in mammary gland development. A manuscript detailing these experiments has been prepared.

The role of galanin in human cancer remains to be investigated, and will be the subject of the final years work. The setback experienced due to unavailable tissue arrays has now been overcome. We are pleased with progress to date, the analysis of the role of galanin has proved to be more time consuming than first envisaged, but our results have shown that galanin's actions are also far more complex than first thought. We have shown for the first time that galanin is a hormone influencing mammary development via multiple mechanisms, which we regard as a major achievement.

Key Research Accomplishments

Analysis of the role of galanin in normal mammary gland development has shown that;
•Galanin controls prolactin release from the pituitary and that prolactin administration partially rescues the mammary phenotype in galanin knockout mice,
•Galanin controls the ratio of phosphorylated to non phosphorylated prolactin and that phosphorylated prolactin inhibits lobuloalveolar development,
•Galanin acts directly on the mammary gland to enhance lobuloalveolar development.

Reportable Outcomes

1. Abstracts of presentations at the following meetings
    a. Australian Society for Medical Research, Leura NSW Nov. 1999
    b. Gordon Conference on Prolactin. Ventura Ca Feb 2000
    c. Keystone Breast and Prostate Meeting, Lake Tahoe NV Mar 2000

2. Manuscripts

stroma. Naylor and Ormandy. To be resubmitted following major modification

•Galanin regulates mammary development by directly controlling lobuloalveolar development and indirectly via control of prolactin phosphorylation and secretion MATTHEW J. NAYLOR[*], RUSSELL C. HOVEY[§], TIMOTHY W. C. HO[†], DAVID WYNICK[¶], BARBARA K. VONDERHAAR[§], AMEAE M. WALKER[†] AND CHRISTOPHER J. ORMANDY[*,**] Provisional authorship.To be submitted shortly.

Conclusions

Galanin modulates mammary gland development indirectly via the prolactin system and directly via yet to be elucidated mechanisms.

So what?

The Nurses Healthy Study (Hankinson 1999) has shown that serum prolactin levels in the top quartile are associated with a 2-3 fold increase in the relative risk of breast cancer, similar to the risk associated with increased serum estrogen levels (Hankinson 1998). The effect of prolactin is independent of estrogen. Thus factors influencing serum prolactin levels can influence susceptibility to breast cancer. Galanin is such a factor.

Galanin's direct effects in breast cancer will be examined in the last year of the grant.

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Hankinson SE. Willett WC. Manson JE. Colditz GA. Hunter DJ. Spiegelman D. Barbieri RL. Speizer FE. Plasma sex steroid hormone levels and risk of breast cancer in postmenopausal women [see comments]. [Journal Article] Journal of the National Cancer Institute. 90(17):1292-9, 1998 Sep 2.

Manuscript

Galanin regulates mammary development by directly controlling lobuloalveolar development and indirectly via control of prolactin phosphorylation and secretion

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Galanin regulation of mammary lobuloalveolar development

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Galanin regulation of mammary lobuloalveolar development

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Abstract

Galanin is a mitogenic neuropeptide involved in neuronal development and neuroendocrine regulation. A number of observations indicate that galanin may also regulate mammary development. For example, galanin is expressed by breast cancer cells and galanin knockout mice fail to lactate. In this report we detail the mechanism of galanin action in the mammary gland. Null mutation of the galanin gene resulted in reduced mammary ductal side branching during puberty and reduced lobuloalveolar development with lactational failure following pregnancy. These mice showed decreased levels of secreted prolactin but also an increased ratio of phosphorylated to unmodified prolactin. While treatment with unmodified prolactin rescued lactational failure in galanin knockout mice, treatment of wildtype mice with a molecular mimic of phosphorylated prolactin inhibited alveolar differentiation and produced lactational failure. These studies show that galanin regulates prolactin driven lobuloalveolar development via the control of prolactin secretion and via the ratio of phosphorylated to unphosporylated prolactin. Galanin and galanin receptor 2 (GALR2) were expressed and differentially regulated in the mammary gland during development. Transplantation of galanin knockout mammary epithelium to normal hosts demonstrated galanin does not have an essential autocrine or paracrine mechanism in the mammary gland. Treatment of whole mammary gland explants in culture with galanin, in addition to lactogenic hormones, resulted in a 3.8 fold increase in the number of lobuloalveoli produced by the lactogenic hormones alone, demonstrating a direct action of galanin. These data demonstrate that galanin acts indirectly via modulation of pituitary prolactin secretion and phosphorylation, as well as directly on the mammary gland to modulate lobuloalveolar development during pregnancy.

Introduction

Postnatal development of the murine mammary gland is systemically controlled by the pituitary-ovarian axis (1). Initially, ductal elongation and birfurcation occurs in response to estrogen and growth hormone during puberty (2). Following puberty ductal outgrowths have filled the mammary fat pad and side branching of ducts occurs in response to progesterone and other factors with each successive estrous cycle (3-5). During pregnancy, under the direction of progesterone and prolactin, alveoli differentiate into lobuloalveoli, a stage of development that is complete following the onset of lactation (4, 6). Following weaning, the mammary gland involutes to a stage where ductal branching closely resembles that seen in virgin mice (7, 8).

These hormones act on cells of epithelial and mesenchymal linages, and induce the autocrine and paracrine action of various signaling molecules (1, 9-13) to direct epithelial morphogenesis (5, 6, 14-18). Over-expression or loss of function of the factors required for normal development results in abberent and failed development or hyperplasia of mammary epithelium (19-23).

Galanin is a 29 amino acid peptide originally isolated from porcine intestine (24) that has been implicated in the control of a number of biological processes including cognition, feeding behaviour, neuroendocrine responses, mitogenesis and nociception (25). Galanin signals through a family of three G-protein-coupled receptors (GALR1-3) (26-28). The generation of mice carrying a loss-of-function mutation of the galanin gene (29) has enabled investigation into the functions of galanin in vivo where it regulates the development of sensory and cholinergic neurons (30, 31). Galanin is a growth factor for

the prolactin-secreting pituitary lactotroph and galanin knockout mice display reduced prolactin levels during pregnancy resulting in lactation failure (29). Galanin also acts as a mitogen for small cell lung cancer, and is amplified in number of breast tumours and breast cancer cell lines where it is steroid-regulated (32-34).

The observation of galanin expression and steroid regulation in breast cancer suggested that galanin may do more during mammary gland development than regulate prolactin secretion. We have utilised galanin knockout mice, combined with mammary transplantation and whole organ culture to determine if galanin is more intimately involved during mammary development.

Methods

Animals.. PRLR and galanin knockout mice (29, 35) used in these studies were 129Ola/129Sv genetic background. $Rag1^{-/-}$ mice (36) on the inbred C57BL/6J background were purchased from Animal Resource Centre, Perth, Australia. All animals were specific pathogen free and housed with food and water *ad libitum* with a 12 hr day/night cycle at 22°C and 80% relative humidity.

mRNA isolation. The 4th inguinal mammary gland frozen in liquid nitrogen before storage at -80°C prior to use. Total RNA, was extracted using TRIZOL Reagent (Gibco BRL) according to the manufacturers instructions.

Reverse Transcription Polymerase Chain Reaction (RT-PCR). First strand cDNA synthesis used avian myleoblastosis transcriptase (Promega) according to the manufacturers instructions. PCR primers for Galanin (Acc No. NM 010253), GALR1 (Acc No. NM 008082), GALR2 (Acc No. NM 010254), GALR3 (Acc No. NM 015738) and GAPDH (Acc No. M32599) were designed on the basis of mismatch to other genes. The following primers were used in this study: (Galanin) mGAL-F1 5'-TGCAGTAAGCGACCATCCAG-3' (forward) and mGAL-R1 5'-AGCACAGGACACACGTGCAC-3' (reverse), (GALR1) mGALR1-F1 5'-CGCCTTCATCTGCAAGTTTA-3' (forward) and mGALR1-R1 5'-CAGGACGGTCTGTGCAGT-3' (reverse), (GALR2) mGALR2-F1 5'-TGCCTTTCCAGGCCACCATC-3' (forward) and mGALR2-R1 5'-GCGTAAGTGGCACGCGTGAG-3' (reverse), (GALR3) GALR3-F1

5'-CCTGGCTCTTTGGGGCTTTCGTG-3' (forward) and GALR3-R1 5'-AGCGCGTAGAGCGCGGCCACTG-3' (reverse), (GAPDH) GAPDH-F1 5'-TGACATCAAGAAGGTGGTGAAGC-3' (forward) and GAPDH-R1 5'-AAGGTGGAAGAGTGGGAGTTGCTG-3' (reverse). PCR reactions were performed in a 50 μL reaction volume containing 5 μL of cDNA, 25 mM $MgCl_2$, 5 μL of 10 x PCR Buffer (containing 100 mM Tris-HCl and 500 mM KCl), 54 pmoles of each primer, 200 μM dNTPs and 3.5 units of Ampli Taq Gold enzyme (Perkin Elmer, CA, USA). The temperature regime consisted of a 94°C 10 min denaturation cycle, followed by 94°C for 25 sec, 58°C for 30 sec, and 72°C for 2 min, for 33 cycles. An elongation step of 72°C for 5 min ended the PCR.

Oligonucleotides for internal hyberisation of PCR products were 5'-AATGGCCACGTAGCGATCCA-3' (GALR1), 5'-GTAGCTGCAGGCTCAGGTTCC-3' (GALR2) and 5'-GTGGCCGTGGTGAGCCTGGCCT-3' (GALR3).

Recombined mammary gland transplantation. Donor mammary tissue (1 mm^3) from galanin wildtype or knockout 12 week old mice was inserted into the excised fat pad of knockout or wildtype 3 week old mice cleared of endogenous epithelium. This recombined mammary epithelium-stroma complex was then grafted between the abdominal cavity and skin, between the 3rd and 4th mammary glands of 3 week old $Rag1^{-/-}$ mice (4). This proceedure resulted in 100% transplant survival with >95% showing ductal outgrowth. Using this method recombinations of mammary epithelium and stroma were produced that allowed deletion the galanin gene from stroma and/or epithelium..

Histological analysis. Mammary whole mounts were made by spreading the gland on a glass slide and fixing in 10% formalin solution. Glands were defatted in acetone before carmine alum (0.2% carmine, 0.5% aluminium sulfate) staining overnight. The whole mount was dehydrated using a graded ethanol series followed by xylene treatment for 60 min and storage and photography in methyl salicylate (37).

Two-dimensional polyacrylamide gel electrophoresis. Following decapitation the anterior pituitary was homogenised and sonicated in 50 mM Tris buffer containing 150 mM NaCl, 5 mM EDTA, 3 mM NaN_3, 0.5% Nonidet P-40 and 10.5 M leupeptin at pH 7.4. The protein was dissolved in urea lysis buffer containing 9 M urea, 5% 2-mercaptoethanol, 4% ampholines pH4-6.5 (Sigma). Electrophoresis was performed according to the method of Ho *et al.* (38). After electrophoresis the gel was silver stained (39), and PRL isoforms identified by immunoblotting (40).

Phosphorylated and unmodified PRL treatment of mice. On the morning of the observation of a vaginal plug, 6-8 week old mice where implanted with a 0.25 µl per hour, 28 day mini-osmotic pump (Alzet) containing either unmodified prolactin or the molecular mimic of phosphorylated PRL S179D (41) to deliver either 0.6 or 1.2 µg per 24 hr. On the first day post partum maternal behaviour of mothers was observed, pups were examined for the presence of milk and glands were taken for histology or western analysis.

Mammary explant culture. Four week old BALB/c mice were implanted with estrogen, progesterone and cholesterol pellets (Innovative Research of America). Following 9 days of treatment, the #4 glands were removed and stretched onto siliconized lens paper and placed into petri dishs containing 2 mL of Waymouths 152/1 media supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), gentamycin sulfate (50 μg/ml), 20 mM HEPES, insulin (5 μg/ml), hydrocortisone (100 ng/ml) and aldosterone (100 ng/ml) to monitor ductal side branching (with and without 100 nM galanin). To assess lobuloalveolar development prolactin (1 μg/ml) was added to the media conditions. Glands were maintained in a tri-gas incubator at 50% O2 and 5% CO2 in air. Media was changed after 24 hr, then every second day for 6 days before morphology and histology were assessed.

Results

Targeted disruption of the galanin gene results in defective mammary development and lactation.

Examination of mammary development in virgin animals at 12 weeks of age, revealed reduced ductal side branching (Fig. 1A-B). On the first day post partum knockout mammary glands showed reduced lobuloalveolar development (Fig. 1D) compared to the normal development seen in wildtype animals (Fig. 1C). Examination of the stomach contents of pups showed that 11 of 12 knockout females were unable to lactate following their first pregnancy, despite normal maternal behaviour. This effect was lost following their second pregnancy. No difference in mammary architecture was observed between wildtype and galanin knockout mice following 5 days of involution (data not shown).

The phosphorylation status of PRL is altered in galanin knockout mice. Galanin has an established role in the modulation of the release of a number of hormones including PRL, a hormone crucial for mammary development and lactogenesis. Recently different biological functions have been identified in vitro for several modification states of PRL. We sought to determine if galanin regulates not only the release of PRL but also the modification state of PRL.

Measurement of the levels of unmodified and phosphorylated PRL were undertaken using pituitaries from both galanin knockout and wildtype male mice. Initially female mice were used but levels were found to vary too widely with estrous cycle to allow accurate measurement. In wildtype male mice 80.6±4.1% of PRL was the unmodified form, while 20.0±1.9% was the phosphorylated form of PRL (Fig. 2). Galanin knockout

mice however had 68.9±3.2% of PRL as the unmodified form and 31.1±2.1% as the phosphorylated form (Fig. 2). The relative ratio of unmodified to phosphorylated PRL, which determines the biological function was 4:1 in wild type mice, compared to 2:1 in knockout mice ($p<0.0001$ Students (unpaired) T-test).

Phosphorylated PRL inhibits lobuloalveolar development and prevents lactation. We sought to determine whether altering the ratio of phosphorylated to unphosphorylated prolactin had an effect on lobuloalveolar development or lactation. Wildtype mice were treated during pregnancy with S179D PRL (41) a molecular mimic of phosphorylated PRL. S179D PRL treatment of wildtype mice completely inhibited lactation and resulted in the death of all pups within 24 hrs of birth (Table 1). S179D PRL treated mice displayed normal maternal behaviour such as nest building and pup retreival, but despite continued sucking the stomachs of pups were empty of milk. Morphological and histological examination of the 4th mammary gland at the first day post partum revealed that lobuloalveolar development was reduced to levels similar to galanin knockout mice (Fig. 3C), and that the differentiation of the alveolar was inhibited compared to control mice (Fig. 3D). These data indicate that regulation of the modification state of PRL can modify prolactin's lactogenic activity. Treatment of galanin knockout mice with S179D PRL did not alter the severity of any of the morphological or lactational defects already present in these animals (Table 1).

Unmodified PRL rescues lactational failure in galanin null mutation mice. Since homogenous disruption of the galanin gene results in decreased levels of plasma prolactin

and an increase in the ratio of phosphorylated to unmodified prolactin, we sought to test whether treatment of galanin knockout mice with unmodified prolactin would rescue the defect in lobuloavleolar development and subsequent lactation failure.

Treatment of galanin knockout mice with either 6 or 12 μg unmodified PRL during pregnancy restored lactation (Table 1). Investigation of mammary gland development following PRL treatment in galanin knockout mice showed that lobuloalveolar development was restored almost to the level observed in control animals (Fig. 3E-F), but histological examination showed that there were fewer lactating ducts and more which retained colostrum in these glands. Thus although prolactin treatment restored development to a level sufficient for pup survival, it did not completely rescue the gland. These data demonstrate that defects in mammary development and lactation observed in galanin null mutation mice are mediated at least in part by PRL deficiency. Treatment with unmodified PRL restores both the reduced plasma levels and the altered ratio of phosphorylated to unmodified PRL. Further experimentation is required to determine if one of these effects is primarily responsible.

Galanin and galanin receptors are differentially expressed in the mammary gland. Expression of galanin and galanin receptors 1-3 was examined by RT-PCR using mammary glands collected at different developmental time points.

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We repeated this analysis in prolactin receptor knockout mice to determine if galanin and its receptors are prolactin regulated. The level of galanin mRNA did not appear to significantly differ between the PRLR knockout and wildtype animals (Fig. 4B). In contrast, GALR2 which is normally expressed in the mammary gland at 12 weeks of age (Fig. 4A) was not expressed in mammary glands from PRLR knockout mice (Fig. 4B). These results indicate that prolactin can increase the sensitivity of the mammary gland to galanin. We also examined PRLR expression in galanin treated glands in organ culture (see below). TO BE COMPLETED PRIOR TO SUBMISSION.

Galanin does not act via an autocrine or paracrine mechanism to regulate mammary development. The expression of galanin and GALR2 in the mammary gland, their differential expression during development and loss in PRLR knockout animals, suggested the possibility of an autocrine or paracrine mechanism of galanin action in the mammary gland. To determine whether mammary galanin production is required for normal development, recombined glands were formed in which the galanin gene was deleted from either the mammary epithelium or stroma, on a background of normal host endocrinology. Deletion of galanin from the stroma or from the epithelium, or from both did not alter mammary architecture observed either during puberty or on the first day post partum (Fig. 5, data not shown). These data demonstrate that mammary galanin production is not required for normal mammary morphology or histology, ruling out an essential autocrine or paracrine role for galanin in the mammary gland.

Galanin can act directly on the mammary gland to induce lobuloalveolar development and proliferation. Next we determined if circulating galanin could act directly on the mammary gland to induce ductal side branching or alveolar proliferation and differentiation. As galanin treatment in vivo may indirectly induce mammary development via endocrine regulation of other growth factors such as PRL, we utilised an in vitro mammary gland explant model of mammogenesis (42).

Ductal side branching similar to that seen during puberty was produced when mammary gland explants were cultured in insulin (I), aldosterone (A) and hydrocortisone (H) (Fig. 6A). The addition of 100 nM galanin to the media did not alter ductal development measured by quantitative morphology, histology and BrdU incorporation (Fig. 6B, data not shown). When PRL was added to the culture media, lobuloalveolar development was observed (Fig. 6C-D), though to a lesser degree than seen during pregnancy *in vivo*. The addition of 100 nM galanin to IAHPRL media resulted in a 3.8 fold increase in the number of lobuloalveoli per gland (33.0±6.1 IAHPRL+Gal vs 8.6±2.1 IAHPRL, P=0.005), causing the glands to better resemble those obtained from *in vivo* pregnancy. These data show that galanin can act directly on the mammary gland to augment lobuloalveolar development, and introduce galanin as a circulating hormone active during this phase of development.

Overall these data show that galanin controls lobuloalveolar development by regulating prolactin secretion and modification by the pituitary, and by acting directly on the mammary gland to also augment the actions of prolactin.

DISCUSSION

This study identifies several novel functions of galanin during mammary development. Galanin acts as a regulator of prolactin signalling via control of prolactin secretion and via regulation of the ratio of phosphorylated to unphosphorylated prolactin isoforms. Loss of prolactin signaling results in the loss of mammary GALR2 expression, demonstrating that galanin can indirectly increase galanin mammary sensitivity via prolactin regulation of its mammary receptor. In addition to these indirect effects, galanin acts directly on the mammary gland to augment the prolactin initiated process of lobuloalveolar development. Secretion of galanin by the mammary epithelium provides an possible endocrine link between mammary epithelial cell number and pituitary prolactin secretion.

These findings establish galanin as an important modifier of prolactin's actions in the mammary gland, and as a new hormone directly involved in lobuloalveolar development. These actions are summerised in Fig 7. Galanin regulates pituitary PRL secretion (29). Work with the PRLR knockout demonstrates that PRL acts indirectly during puberty to control ductal side branching via regulation of ovarian progesterone. Estrogen levels are also decreased in PRLR knockout mice but not sufficiently to alter ductal elongatrion and bifurcation. Development in mammary transplants from this model stall following alveolar bud formation, demonstrating that prolactin acts directly on the mammary gland to initiate lobuloalvelor development (6, Naylor and Ormandy unpublished data). The regulatory role played by galanin in this process is demonstrated by the rescue of lactational failure in galanin knockout mice by treatment with unmodified PRL.

Galanin also regulates PRL function by dramatically altering the ratio of PRL isoforms, shown in Figure 7 as negative regulation of prolactin phosphorylation. Posttranslational modification of hormones serve as a mechanism of regulating hormone action. Several variants of PRL exist that are the result of alternate splicing, proteolytic cleavage, phosphorylation, glycosylation, dimerisation and polymerization (49). The biological significance of the variant forms of prolactin is only beginning to be established but it appears that most forms of PRL modification serve to inhibit PRL function. A molecular mimic of phosphorylated PRL (S179D) was used to determine the biological significance of phosphorylated PRL during murine mammogenesis. Biological activity of S179D mimics that of naturally phosphorylated PRL (41). S179D PRL treatment resulted in the inhibition of unmodified PRL biological function in the mammary gland, demonstrating a biological function of phosphorylated PRL and identifying a second mechanism of galanin control over PRL function. Phosphorylated PRL may regulate unmodified PRL action as either a PRLR antagonist (52), or by downregulating unmodified PRL in an autocrine manner (53).

In addition to controlling mammary development via regulation of PRL, galanin also acts directly on the mammary gland to induce lobuloalveolar development. Galanin probably acts via GALR2 as this is the only known galanin receptor expressed during pregnancy and mice with a null mutation of GALR1 demonstrate normal mammary development and lactation (MJN, CJO, Arie Jacoby and Tiina Iismaa, data not shown).

The induction of alveolar proliferation and differentiation by galanin raises the question of whether galanin may also act as a mitogen for breast cancer. In small cell lung cancer galanin has a demonstrated role as a mitogen (34). We have previously demonstrated that

galanin is expressed in a number of human breast cancer cell lines and that expression is steriod regulated (32). All 3 galanin receptors have varied expression in the same panel of human breast cancer cell lines (MJN and CJO, unpublished result). The expression of all 3 galanin receptors is interesting as only GALR2 is expressed by normal mammary epithelial cells. The role of galanin as a mitogen for breast cancer cells is currently under investigation, however to date we have been unable to demonstrate a mitogenic effect of galanin alone.

Targeted disruption of components of the PRL signalling cascade have demonstrated the importance of this pathway in mammopoiesis and lactation (35, 43, 44). Likewise, we and others have also recently demonstrated that absolute levels of positive and negative modulators of the prolactin pathway are essential for normal mammary development (Lindeman *et al.*, 2001). In this study we have demonstrated a novel role for galanin acting as a positive regulator of PRL signalling via regulation of PRL secretion and modification. The direct action of galanin on the mammary gland demonstrates a novel role for galanin as a hormone regulating mammary development. It will be interesting to determine which genes are directly regulated by galanin in the mammary gland, further dissecting the complex regulation of mammopoiesis.

Acknowledgments

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Table 1. Effects of galanin, prolactin and phosphorylated prolactin on lactation

Mouse	Treatment	N	Lactation (N)	No lactation (N)	Percentage lactating
Galanin -/-	nil	12	1	11	8.3***
+/+	nil	15	15	nil	100
Galanin -/-	PRL*	6	5	1	83.3
Galanin -/-	S179D*	5	1	4	20***
+/+	S179D*	4	1	3	25***

* mini-osmotic pump delivering 0.6 or 1.2 μg/24h/mouse

*** $P < 0.0001$ versus wildtype (+/+) control.

Figure legend

Fig. 1.

Morphological defects of galanin knockout mice during puberty and lactation. Carmine stained whole mounts. (A) wildtype, virgin, 12 weeks. (B) galanin knockout, virgin 12 weeks, reduced ductal side branching evident. (C) wildtype, lactation, first day post partum. (D) galanin knockout, lactation, first day post partum, reduced lobuloalveolar development.

Fig. 2.

Levels of prolactin isoforms in the pituitaries of male galanin knockout (A) and control mice (B). Stained 2-D gel analysis of anterior pituitary prolactin isoforms (U-unmodified prolactin, P-phosphorylated prolactin).

Fig. 3.

Administration of unmodified and phosphorylated prolactin to control and galanin knockout mice. (A,C,E,G) Carmine stained whole mount analysis. (B,D,F,H) Haematoxylin and eosin stained 5 μm sections. (A,B) Wildtype mice treated with saline during pregnancy, normal development and lactation. (C,D) Wildtype mice treated with S179D, lobuloalveolar proliferation normal, differentiation not complete as lactation failed. (E,F) Galanin knockout mice treated with unmodified PRL, lactation and lobuloalveolar development restored (G,H) Galanin knockout mice untreated, reduced lobuloalveolar proliferation and failure of differentiation. Arrows indicate ducts.

Fig. 4.

Differential expression of galanin and galanin receptor mRNA during mammary development. (A) Expression of galanin and galanin receptors at varying developmental time points by hybridization of RT-PCR products. (B) PRLR regulation of mammary GALR2. GALR2 mRNA was not detected in mammary glands from PRLR -/- mice at 12 weeks of age.

Fig. 5.

Galanin does not act via autocrine or paracrine mechanisms to regulate mammary gland development. Carmine stained whole mounts of galanin -/- (B,D) & +/+ (A,C) epithelium transplanted into the fat pad of $Rag1^{-/-}$ mice cleared of endogenous epithelium. (A,B) virgin (C,D) 1st day post partum.

Fig. 6.

Galanin acts directly on the mammary gland to induce lobuloalveolar development. In vitro mammary gland explants treated with (A) IAH, (B) IAH + galanin, (C) IAH + PRL and (D) IAH + PRL + galanin. Arrows indicate lobuloalveoli.

Fig. 7.

Summary of the role of galanin in mammary gland development. The stages of mammary gland development are shown schematically with causitive reproductive events indicated above and descriptions of subsequent morphological changes given above each open

arrow. Hormone secretion is shown by solid arrows. Regulatory influences on hormones or morphology are indicated by dashed lines that are positive (arrow heads) or negative (lines).

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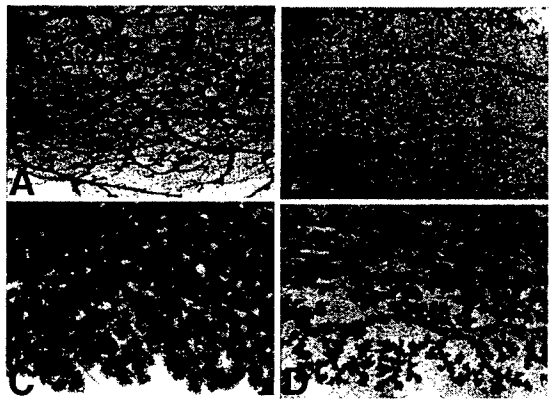

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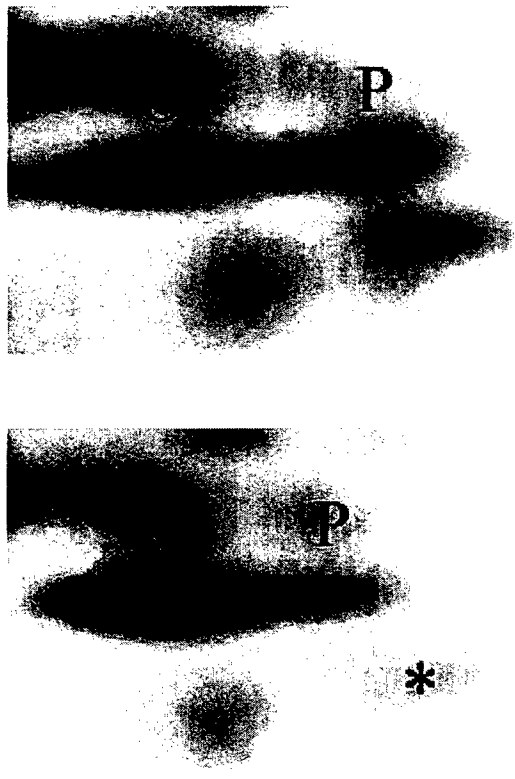

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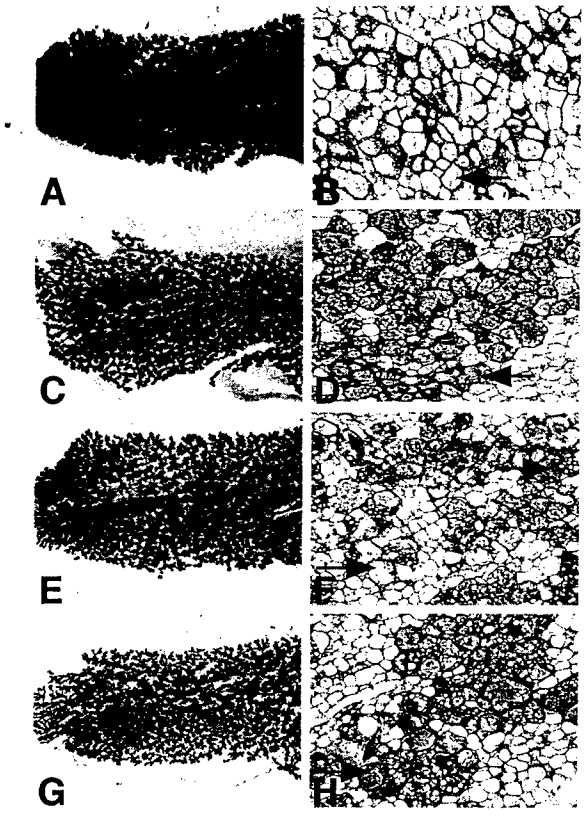

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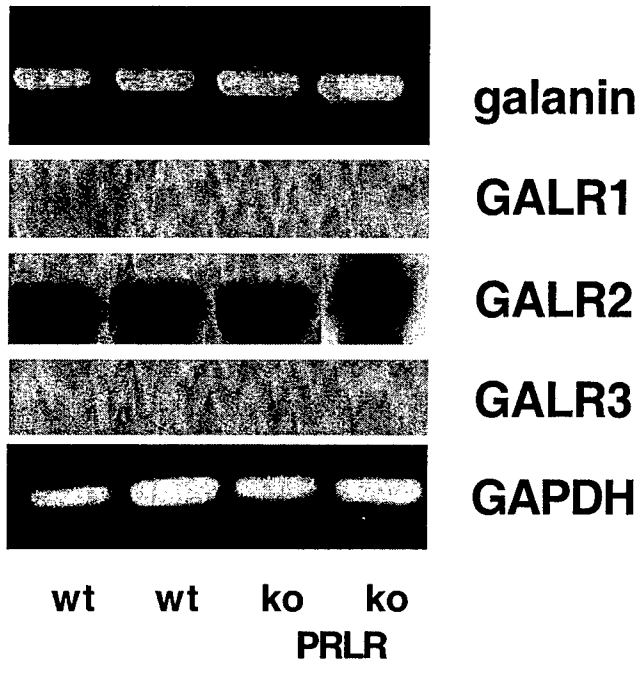


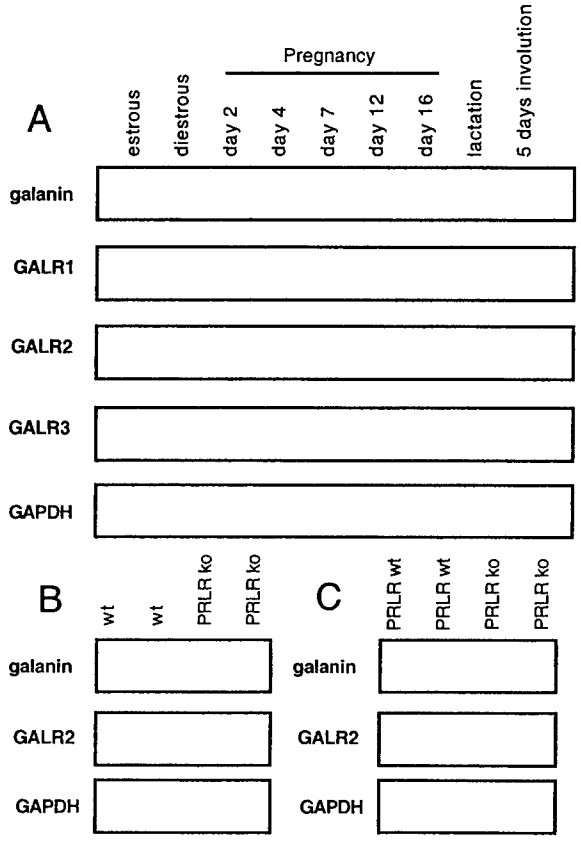
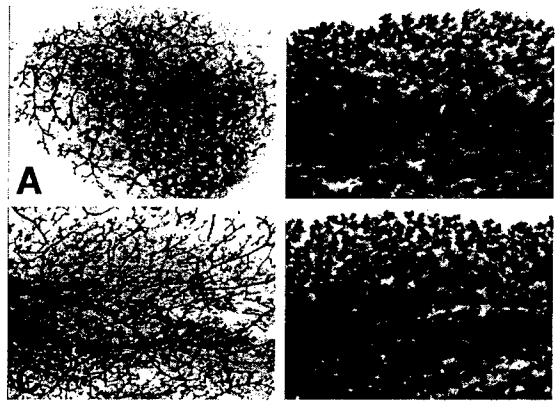


Figure 4



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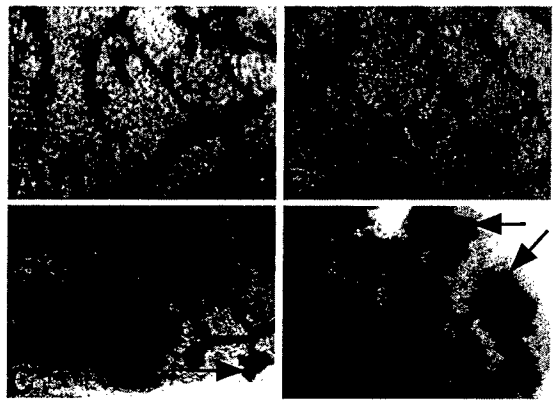

Figure 6

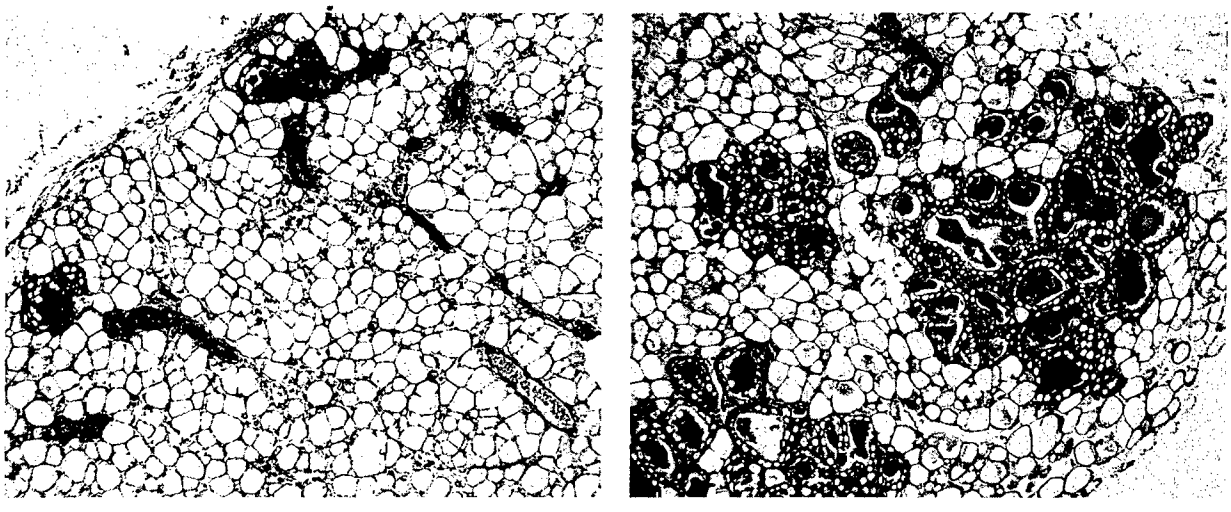

Figure 6 (Continued)

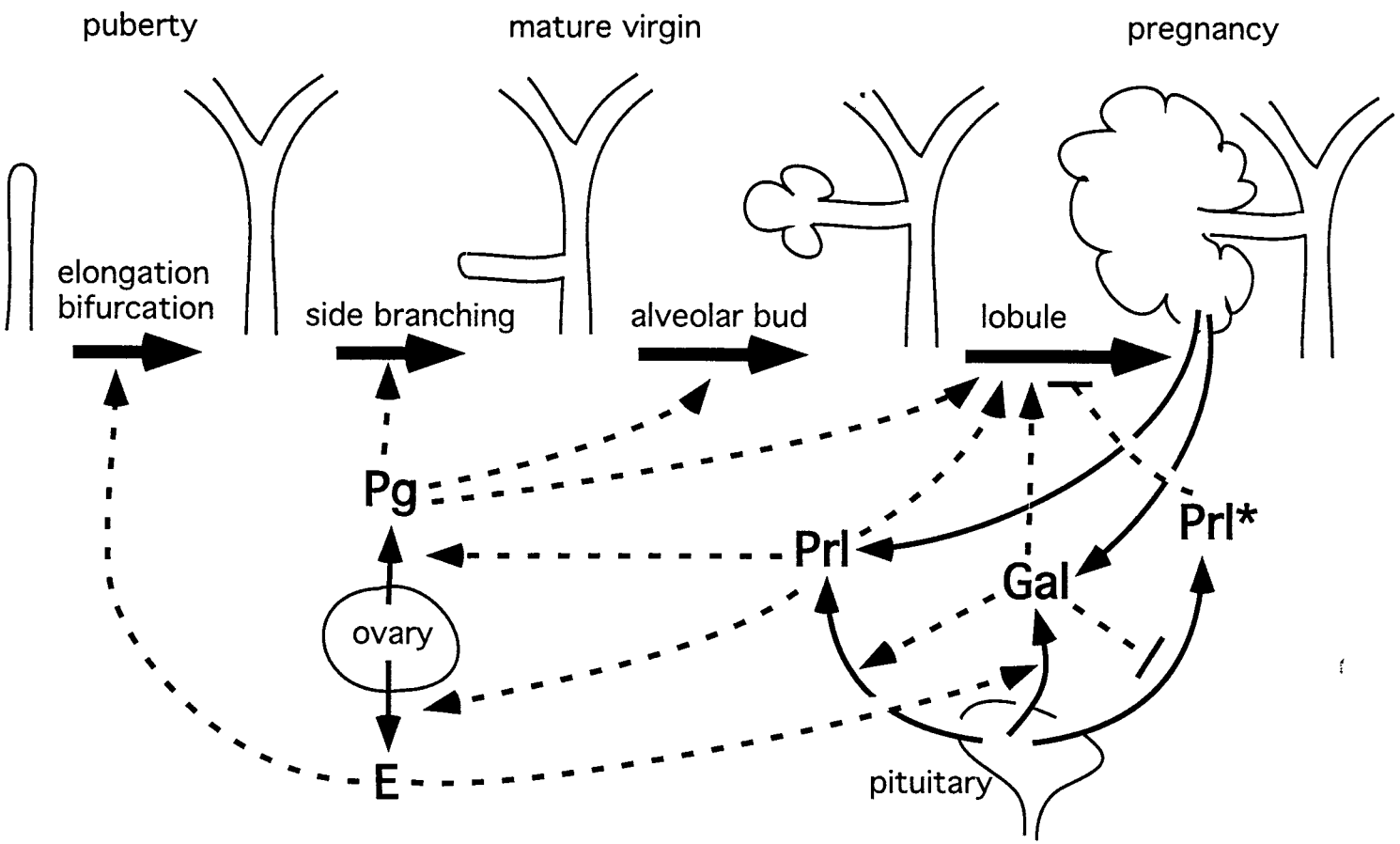
puberty
mature virgin
pregnancy
elongation
bifurcation
side branching
alveolar bud
lobule
Pg
Prl
Prl*
Gal
ovary
E
pituitary

Figure 7

Mouse	Treatment	N	Lactation (N)	No lactation (N) lactating	Percentage
Galn -/-	nil	12	1	11	8.3***
+/+	nil	15	15	nil	100
Galn -/-	PRL*	6	5	1	83.3
Galn -/-	S179D*	5	1	4	20***
+/+	S179D*	4	1	3	25***

* mini-osmotic pump delivering 0.6 or 1.2 μg/24h/mouse
*** $P < 0.0001$ versus wildtype (+/+) control.

Table 1

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MD 21702-5012

REPLY TO
ATTENTION OF

MCMR-RMI-S (70-1y) 15 May 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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